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Long-term biological variability and the generation of a new reference interval for plasma N-terminal pro-B-type natriuretic peptide in Labrador retrievers

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OBJECTIVES: First, to investigate the biological variability of N-terminal pro-B-type natriuretic peptide (NT-proBNP) in healthy Labrador retrievers and compare this with current laboratory recommendations for dilated cardiomyopathy screening. Second, to calculate a breed-specific reference interval and validate it in a retrospective cohort.

MATERIALS AND METHODS: Plasma NT-proBNP was measured in 51 clinically healthy Labrador retrievers at 0, 2, 4, 6 and 8 weeks. Coefficient of variation for individual dogs over time, the coefficient of variation for the group at each time point and the index of individuality were calculated. A reference interval was derived and tested on a clinical dataset available from four UK cardiology referral centres.

RESULTS: Median NT-proBNP was 865 pmol/L (315 to 2064 pmol/L). Mean individual coefficient of variation was 19% (95% CI: 16 to 21%) and group coefficient of variation was 43% (95% CI: 41 to 46%), with index of individuality at 0.44. The breed-specific reference interval was 275 to 2100 pmol/L. In the validation group, 93% of NT-proBNP measurements from healthy dogs were within the reference interval. NT-proBNP measurements exceeded the reference interval in 82% of dogs with dilated cardiomyopathy. The upper bound of the reference interval (2100 pmol/L) had a positive predictive value of 90% and a negative predictive value of 87% for identification of dilated cardiomyopathy in this population.

CLINICAL SIGNIFICANCE: Breed-specific reference intervals might improve the diagnostic accuracy of NT-proBNP measurement. Applying the currently recommended general cut-off value to Labradors is likely to result in frequent false positives and diagnosis would be improved by application of the new breed-specific reference interval calculated here.

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INTRODUCTION

N-terminal pro-hormone of B-type natriuretic peptide (NT-proBNP) is released by the atrial and ventricular myocardium as a result of increased stretch or stress on the heart. Plasma NT-proBNP is higher in dogs with heart failure, as well as in dogs with occult heart disease (Prosek *et al.* 2007, Boswood *et al.* 2008, Fox *et al.* 2009, Singletary *et al.* 2012). Occult dilated cardiomyopathy (DCM) can be difficult to detect without echocardiography, as only an estimated 33% of affected dogs have an audible heart murmur (Martin *et al.* 2009). Given the previously identified benefit of pimobendan in Doberman pinschers with pre-clinical DCM (Summerfield *et al.* 2012), many veterinarians wish to detect occult disease in at-risk individuals. Rather than perform echocardiography in every at-risk dog, screening using plasma NT-proBNP measurement is an attractive proposition for veterinarians in general practice. Based on a study evaluating NT-proBNP in Doberman pinschers with occult DCM (Wess *et al.* 2011) laboratory advice suggests that, in an at-risk breed of dog, plasma NT-proBNP ≥ 900 pmol/L is compatible with clinically meaningful heart disease (IDEXX Laboratories interpretative criteria; https://idexxcom-live-b02da1e51e754c9cb292133b-9c56c33.aldryn-media.com/filer_public/26/89/26893bee-e598-479f-ba85-b0084b2ff542/cardiopet-interpretive-criteria-canine.pdf, accessed August 23, 2018), but was based on a previous-generation assay.

Despite its potential utility for screening purposes, NT-proBNP is subject to day-to-day variability, which could potentially generate false-positive and false-negative test results in patients in which measurements are close to the diagnostic cut-off. Previous studies investigating the biological variability of NT-proBNP have suggested approximately 50% variability over a 6 to 7-week period (Ruau *et al.* 2015, Winter *et al.* 2017). However, these data were obtained from relatively small samples containing a mixture of dog breeds, variable environments and inconsistent sample handling. Additionally, breed differences in plasma NT-proBNP have been identified in healthy dogs (Sjöstrand *et al.* 2014). Some breeds, including the Labrador retriever, were reported to have median NT-proBNP values which exceeded 900 pmol/L (Sjöstrand *et al.* 2014). Both biological variability and breed differences suggest that the results of NT-proBNP screening for occult DCM in non-Doberman breeds should be interpreted with caution.

The Labrador retriever is considered predisposed to DCM (Martin *et al.* 2009) and is a common breed in the UK; in 2017, it was the most frequently registered breed of dog with the Kennel Club (IDEXX Laboratories interpretative criteria; https://idexxcom-live-b02da1e51e754c9cb292133b-9c56c33.aldryn-media.com/filer_public/26/89/26893bee-e598-479f-ba85-b0084b2ff542/cardiopet-interpretive-criteria-canine.pdf, accessed August 23, 2018). It is therefore reasonable to assume that UK-based practitioners might use plasma NT-proBNP measurement as a screening test for occult DCM. Thus, a better understanding of biological variability and a breed-specific reference interval for Labradors is likely to be clinically useful.

The aim of this study was to assess the biological variation in plasma NT-proBNP over 32 weeks in a large cohort of clinically

healthy, echocardiographically normal Labrador retrievers, for which the environment and exercise regime were standardised. In addition, we aimed to derive and test a novel, breed-specific reference interval for plasma NT-proBNP in Labradors.

Our hypotheses were: (1) the mean plasma NT-proBNP in clinically healthy Labradors exceeds 900 pmol/L, (2) serial measurement of plasma NT-proBNP in Labradors will reveal clinically-relevant biological variability over 32 weeks, and (3) a newly-derived reference interval from this population of healthy Labradors more accurately identifies dogs with occult DCM than a cut-off value of 900 pmol/L.

MATERIALS AND METHODS

Evaluation of biological variability and reference interval

The study protocol was approved by the Waltham Animal Welfare and Ethical Review Board, and carried out under the authority of the Animals (Scientific Procedures) Act 1986.

Healthy adult Labrador retrievers ($n = 51$), fed nutritionally complete diets (Allaway *et al.* 2017, Harrison *et al.* 2017), were housed at the Waltham Centre for Pet Nutrition, UK. The environment and exercise regime were standardised for all dogs. Prior to enrolment, dogs were habituated to all study procedures, including echocardiography and blood sampling. At enrolment, all dogs underwent complete physical examination, 2-dimensional and Doppler echocardiography, complete blood count and serum biochemical profile. Dogs were eligible to proceed with the study protocol if considered clinically healthy (no evidence of cardiac or systemic disease detected on the aforementioned tests).

All echocardiographic examinations were carried out by a board-certified veterinary cardiologist (KB) using a cardiovascular ultrasound platform (GE Vivid I, with a 5S phased-array transducer; GE Healthcare, Hatfield, UK). Transthoracic 2-dimensional and Doppler echocardiographic studies were performed from both sides of the thorax, alongside a simultaneous electrocardiogram (ECG) recording. Dogs were considered echocardiographically normal if they met all of the following criteria: left atrial-to-aortic root ratio (LA:Ao) < 1.5 measured in the right parasternal short-axis window, optimised for the heart base; left ventricular end-systolic volume indexed to body surface area (ESVi; measured from a right parasternal long-axis window) < 30 mL/m²; ejection fraction (EF) $> 45\%$; left ventricular internal diameter in diastole normalised for body weight (LVIDd-N, measured using M-mode cursor bisecting the left ventricle in a right parasternal short-axis window optimised for the papillary muscles and chordae tendineae) < 1.7 and diastolic left ventricular wall thickness were within reference intervals for body weight using an allometric scaling method (Cornell *et al.* 2004). Aortic and pulmonic Doppler flow profiles were obtained and considered normal if aortic maximum velocity was < 1.7 m/second (from a subcostal view) and pulmonic maximum velocity was < 1.2 m/second (from the right parasternal short-axis window).

Fasted blood samples were collected from the jugular vein into EDTA containers. Samples were obtained at weeks 0, 8, 16, 24 and

32. Sampling was performed at the same time of day (within a 2-hour window) and EDTA samples were centrifuged (1000 g, 10 minutes, 4°C) to separate plasma. All plasma samples (500 µL) were prepared within 1 hour of blood collection and stored in polypropylene tubes at -80°C. After shipping on dry ice to a reference laboratory (IDEXX Laboratories, Wetherby, UK), batch analysis of NT-proBNP was performed in duplicate with the second-generation canine Cardiopet® proBNP ELISA (detection limits 16 to 10,000 pmol/L).

Validation of the newly derived reference interval

Clinical records of four cardiology referral centres in the UK (Langford Vets, Royal Veterinary College, Heart Vets and Lumbry Park Veterinary Specialists) were retrospectively searched for client-owned Labradors with results of echocardiography and plasma NT-proBNP measurement available. Dogs were classified according to the diagnosis made by a cardiology diplomate as apparently healthy (no heart disease nor significant systemic disease), or as having cardiac disease. Within the cardiac disease group, dogs were additionally sub-classified as having been diagnosed with DCM or other cardiac diseases. Other data collected were plasma NT-proBNP concentration, echocardiographic diagnosis, age, sex, neutering status and bodyweight. NT-proBNP concentrations were compared between groups and with the prospectively derived reference interval to evaluate potential clinical utility.

Statistical analysis

Statistical analysis was performed using commercially available software (SPSS 22 for Windows, IBM GraphPad Prism GraphPad Software, La Jolla, California). Data were assessed for normality both graphically and using Shapiro Wilk tests. Descriptive statistics were reported as mean (\pm standard deviation) for normally distributed data and as the median (range) for non-normally distributed data. Outliers were determined using the Tukey Method: defining limits on the sample values that were a factor 1.5 of the interquartile range below the 25th or above the 75th percentile. Statistical significance for all tests was set at $P < 0.05$. The maximum, minimum and mean NT-proBNP values and measures of variability were calculated.

Variability for plasma NT-proBNP values was assessed by calculating the group coefficient of variation (CV_G ; corresponding to the variability between dogs at each time-point) and the individual coefficient of variation (CV_I ; corresponding to the variability within each dog over all time-points). The CV_I was calculated using the square root of the variance (standard deviation) of individual dog over all time points, divided by the mean value of individual dog over all time points. The result was reported as the mean CV_I for all dogs. The CV_G was calculated using the square root of the variance of all dogs at a single time point, divided by the mean value of all dogs at a single time point. This was done at the first visit, at 2, 4, 6 and 8 months. The result was reported as the mean CV_G for all time-points. The index of individuality (IOI) was calculated as the CV_I : CV_G ratio (Fraser & Harris 1989, Fraser 2001). For derivation of the reference interval, a single sample was randomly selected from each dog using an online random number generator (random.org). The reference interval calculation was subsequently determined using a robust

bi-weight quantile method computed on the reflected sample (Analyse-it Software, Ltd).

Using retrospective clinical data, positive and negative predictive values (PPV and NPV, respectively) for the detection of DCM were calculated using the upper bound of the derived reference interval. PPV was determined as the ratio between true positives and the total number of positives, whereas the NPV was determined as the ratio between true negatives and the total number of negatives. An online statistics calculator (The Chinese University of Hong Kong, <https://www2.ccrb.cuhk.edu.hk/stat/confidence%20interval/Diagnostic%20Statistic.htm#Help>) was used to formulate 95% confidence intervals (95% CI) for PPV and NPV, because of the relatively small validation cohort.

RESULTS

Biological variability of NT-proBNP and derivation of a breed-specific reference interval

A group of 62 Labradors underwent echocardiography training over a 2-month period, which involved them presenting themselves and lying in right and left lateral recumbency for echocardiography with minimal restraint. All dogs successfully completed this training, but 10 dogs were excluded from the study because behavioural assessment indicated that the dogs could not undergo blood sampling without undue stress. Serial measurement of NT-proBNP was performed in 52 dogs. Analysis identified one dog as an outlier, which was removed from the statistical analysis. Of the 51 dogs analysed, median age was 5.2 years (2.3 to 8.2 years). More female dogs ($n = 32$) were recruited than males ($n = 19$). All animals were neutered. The median weight was 26.6 kg (23.5 to 29.7 kg). One other dog was removed from the variability analysis because only one sample was collected. Data from 50 dogs were included in the analysis of biological variability.

A total of 242 plasma samples were obtained. All five planned plasma samples were collected from 44 dogs, four of five were collected in four dogs, and in one dog each, three, two and one

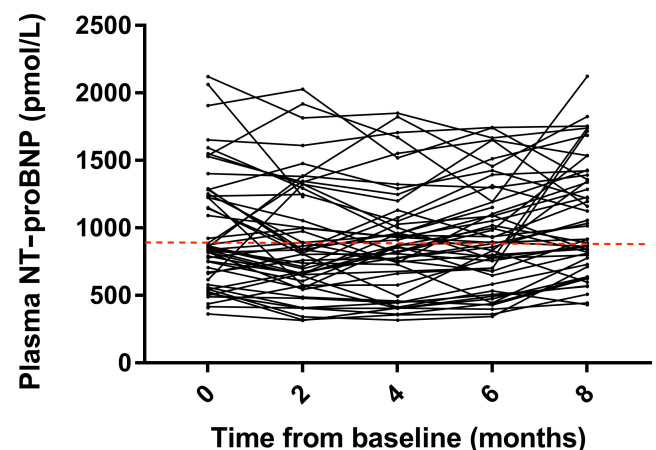


FIG 1. Serial measurements of plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) over a 32 week period in 51 healthy Labrador retrievers. Each solid line represents an individual dog. The dotted line represents the laboratory recommended cut-off for dilated cardiomyopathy (DCM) screening at 900 pmol/L

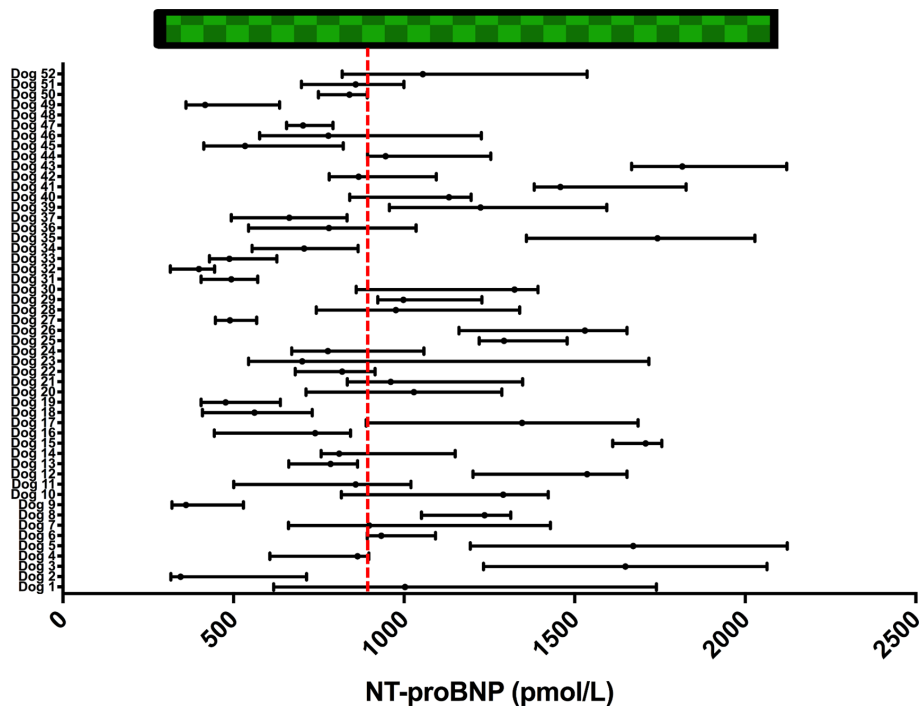


FIG 2. Mean and range plots displaying the degree of biological variability of plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) measurements in healthy Labradors over 32 weeks, and the reference interval derived from these data. The calculated reference interval was 275 to 2100 pmol/L. The laboratory recommended upper reference limit of 900 pmol/L is illustrated by a vertical dashed line

sample only were collected. Median NT-proBNP was 865 pmol/L (315 to 2064 pmol/L). In healthy Labradors, 38.8% (94/242) of plasma samples obtained over the 32-week study period exceeded 900 pmol/L. At least one NT-proBNP measurement >900 pmol/L was recorded in 64.7% of dogs during the study period (Fig 1). The calculated CV_I was 19% (95% CI: 17 to 22), and CV_G was 43% (95% CI: 41 to 46%). IOI was 0.44. The reference interval derived from this group of healthy Labrador retrievers was 275 to 2100 pmol/L (95% CI for lower value 120 to 341, for upper value 1782 to 2376) (Fig 2).

Validation of the breed-specific reference interval

Clinical record searches from the four referral centres identified 34 suitable case records. Cardiac disease was diagnosed by a cardiologist in 20 dogs, with 14 dogs having no evidence of heart disease (Table 1). Population characteristics are presented in Table 2.

In the normal group, median plasma NT-proBNP was 1238 pmol/L (760 to 2307 pmol/L) and 13/14 (93%) of results were within the breed-specific reference interval. In dogs with any cardiac disease, median plasma NT-proBNP was 2927 pmol/L (449 to 10,000 pmol/L) and 14/20 (73%) of measurements were >2100 pmol/L. In the sub-group of Labradors with DCM, median plasma NT-proBNP was 2934 pmol/L (1716 to 10,000 pmol/L) and 9/11 (82%) of measurements were >2100 pmol/L (Fig 3).

Considering only dogs with DCM *versus* normal dogs, using the laboratory recommended cut-off value of >900 pmol/L yielded a PPV of 46% (95% CI: 26 to 66) and NPV of 100% (95% CI: 100 to 100) for identification of DCM. Using the upper bound of the new breed-specific reference interval as a

Table 1. Cardiac diagnoses of Labrador retrievers included in the retrospective validation dataset

| Diagnosis | Number of dogs |
|--|----------------|
| No cardiac abnormalities detected (apparently healthy) | 14 |
| Dilated cardiomyopathy | 11 |
| Isolated arrhythmia | |
| Third degree atrioventricular block | 3 |
| Isorhythmic atrioventricular dissociation | 1 |
| Infiltration or inflammation | 2 |
| Mitral valve disease | 1 |
| Pericarditis | 1 |
| Tricuspid valve dysplasia | 1 |

cut-off (>2100 pmol/L) yielded a PPV of 90% (95% CI: 71 to 100) and an NPV of 87% (95% CI: 69 to 100) for identification of DCM.

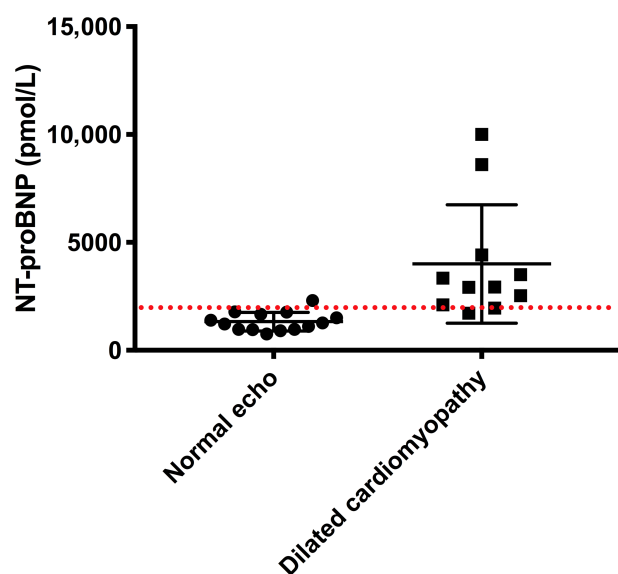
DISCUSSION

The results of this study suggest that the laboratory recommended cut-off for NT-proBNP of 900 pmol/L for DCM screening in Labrador retrievers results in unacceptably low diagnostic accuracy and a Labrador-specific reference interval should be adopted. Not only do apparently healthy Labradors appear to have a higher plasma NT-proBNP than other breeds, as described previously (Sjöstrand *et al.* 2014), but they exhibit clinically important long-term biological variability. Whilst a relatively low cut-off value will strengthen the negative predictive value, frequent false-positive results may be unacceptable in a practical screening programme

Table 2. Population characteristics of client-owned Labradors in the validation cohort

| Group | n | Weight (kg), median (range) | Age (years), median (range) | Male | Plasma NT-proBNP (pmol/L), median (range) |
|---------------------|----|-----------------------------|-----------------------------|-------|---|
| Normal | 14 | 30.9 (24.7 to 41) | 8 (2 to 13) | 7/14 | 1238.5 (760 to 2307) |
| All cardiac disease | 20 | 30.5 (26.2 to 48.7) | 7.5 (1 to 13) | 16/19 | 2927 (449 to 10,000) |
| DCM | 11 | 30 (26.6 to 48) | 7.5 (1 to 13) | 8/11 | 2934 (1716 to 10,000) |

DCM Dilated cardiomyopathy, NT-proBNP N-terminal pro-B-type natriuretic peptide.

**FIG 3. Compound dot plots displaying plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) measurements in dogs with normal cardiac examinations (apparently healthy, n = 14) and dogs with dilated cardiomyopathy (n = 11). The upper limit of a proposed breed-specific reference interval is illustrated by the horizontal dashed line (2100 pmol/L)**

for animals with no current clinical signs of disease. We therefore suggest that a new reference interval of 275 to 2100 pmol/L is used by practitioners aiming to identify which Labradors should undergo echocardiography for DCM screening.

In our cohort of clinically healthy Labradors, we identified a high degree of biological variability in plasma NT-proBNP measurements. The CV_I (within subject biological variability over time) was 19% and the CV_G (biological variability within the group at each time point) was 43%. This degree of variability is in agreement with previous studies reported from cohorts of dogs made up of a mixture of breeds (Ruau *et al.* 2015, Winter *et al.* 2017), but our study featured a larger sample size over a longer time frame, and a homogeneous population of dogs: same breed, uniform diet, environment and exercise regime, fed to maintain an ideal body condition score and a standardised sampling time within the day. This excludes some of the innate variation in plasma NT-proBNP measurements between breeds (Sjöstrand *et al.* 2014), different sampling times (diurnal variation) (Goetze *et al.* 2010) and differences in exercise regimens (Hunt *et al.* 2018, Park *et al.* 2018).

The IOI arising from this study was low, at 0.44. This is in accordance with a previous report of low IOI for plasma NT-proBNP measurements (Winter *et al.* 2017). In tests with low IOI (<0.6), there is low individual variability relative to group

variability (in the present study, 19% *versus* 43%). In this situation, clinically meaningful changes in an individual over time may be masked by group variation. However, animals with values above the upper bound of the reference interval are likely to be truly abnormal (Fraser 2001). This is supported by a strong PPV of 90% for DCM screening in our validation cohort. However, it is worth considering that some dogs within the population may be abnormal at a lower plasma NT-proBNP; this is supported by the data having a low IOI. In tests exhibiting this, an upward trend over time may suggest that an individual is developing an abnormality despite being within the overall population reference interval (Fraser 2001). Therefore, in a Labrador for which clinicians have a high index of suspicion for cardiac disease (for instance, a close family history), an upward trend over time (we suggest three consecutive samples over 1 to 2 months, given the degree of variability in our data) might suggest that echocardiography is indicated, even if NT-proBNP measurements remain within the reference interval.

Our study is subject to several limitations. First, circulating plasma NT-proBNP concentrations can be affected by, amongst other things, azotaemia, pulmonary hypertension, sepsis, or systemic hypertension (Oyama *et al.* 2013). Whilst we did our best to exclude dogs possibly affected by these conditions from both data sets, it is possible that affected dogs were inadvertently included. Secondly, the derivation and validation populations of dogs in this study were not identical, with the validation cohort including some older dogs that might be more likely to have comorbidities. In humans, an association of increasing age with the inability to lower NT-proBNP has been shown (Richards 2016). This relationship appears to reflect the interaction of age with the total burden of comorbidities rather than reflecting an intrinsic characteristic of age *per se* (Richards 2016). Also, in the validation cohort, samples may have been handled differently (albeit all submitted to the same laboratory), and the population would have been less homogeneous than the group of dogs used to derive the reference interval. Some may also have been in congestive heart failure at the time of NT-proBNP measurement, which would not reflect the use of the test by a practitioner as a screening tool in an apparently healthy dog. There were also only a relatively small number of cases available for inclusion. Whilst the heterogeneity more accurately represents client-owned dogs presenting to clinical practice, this and the small sample size might have influenced the calculated PPV and NPV. In addition, all dogs were referred to cardiologists, so might not represent the wider general practice population of dogs. Also, in the derivation cohort of apparently healthy dogs, individuals underwent echocardiography at baseline only. Although an ECG was recorded

during the echocardiogram and showed normal sinus rhythm for all the dogs, a Holter (24 hours ECG) would have been necessary to further confirm the absence of some arrhythmias over a longer time period. It is therefore possible that some dogs might have developed undetected cardiac disease over the 32 week-long study period, or have suffered unacknowledged cardiac arrhythmias. Although unlikely, this would have skewed our assessment of biological variability. It is worth considering that in Doberman pinschers with various stages of DCM, diagnosed based on echocardiographic and 24 h Holter ECG data, no significant difference in NT-proBNP was identified between unaffected dogs and those with a diagnosis based on Holter only (*i.e.* normal echocardiogram) (Wess *et al.* 2011). Therefore, our proposed reference interval should be considered as an additional screening tool for dogs with DCM detectable on imaging, and may miss those with arrhythmias only. Finally, the apparently healthy dogs were all kept in a controlled kennel environment that may not be entirely representative of a client-owned dog population.

In conclusion, clinically important biological variability of plasma NT-proBNP was identified over 32 weeks in a relatively homogeneous group of Labrador retrievers, which frequently would lead to a false-positive result in DCM screening using a cut-off of 900 pmol/L. For this reason, we recommend a new, breed-specific, upper bound of 2100 pmol/L. Further investigation for heart disease should be recommended in Labrador retriever in which plasma NT-proBNP measurement exceeds 2100 pmol/L. Trends over time should be monitored in high-risk individual dogs.

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Conflict of interest

DA and MH are employees of MARS PetCare Ltd. MJH, JRP, KB and DD have all previously either received research funding or discounted analysis prices for research from IDEXX Laboratories Ltd.

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